BIOLUMINESCENCE

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INTRODUCTION

Numerous studies during the past fifty years on luminous organisms have established beyond doubt that the necessary energy for light production is yielded by chemical reactions (1, 2). Bioluminescence is a special form of chemiluminescence. The nature of the biochemical reactions which result in an electronically excited state of some molecule which subsequently emits light is, therefore, the central problem in bioluminescence. The present review will be concerned primarily with the nature of these biochemical reactions and the structure of the light emitting molecule. Certain aspects of the physiology of luminous bacteria will also be discussed.

Luminescence is distributed widely in the animal and plant kingdoms, and Harvey has recently summarized most of this information in his book (2). The first definitive experiment regarding the nature of the components necessary for light production was reported by Dubois in 1885 (3). He found that the luminous organs of *Pyrophorus*, a luminous beetle, would cease to emit light if immersed in hot water. He found, however, that a cold water extract which had ceased to luminesce could be stimulated to emit light by adding the hot water extract. On the basis of this type of experiment, Dubois proposed the theory that there was, in the hot water extract, a substance stable to heat which was destroyed during its luminescent oxidation through the action of a catalyst present in the cold water extract. He named the heat stable material luciferine and the enzyme which catalyzed its oxidation luciferase. This early work of Dubois was greatly extended and clarified through the efforts of E. Newton Harvey and his associates at Princeton (2). The luciferin-luciferase reaction has been demonstrated in a number of luminous species while a variety of physical and chemical studies has been performed on the luminous extracts of the ostracod crustacean, *Cypridina hilgendorfii*. Subsequent studies on the nature of the luciferin in other forms led, however, to the concept that the first rate limiting factor in cold water extracts was not necessarily the oxidizable substrate. In 1947 McElroy (4) demonstrated that the loss of light emission in cold water extracts of fireflies could be restored by the addition of adenosine triphosphate. Later ex-

tensive studies (5, 6, 7) to be discussed below established the presence of a second factor which logically could be called firefly luciferin. Although this factor is destroyed during luminescent oxidation, it does not become rate limiting in the cold water extracts except for a few species of fireflies. Recently Strehler (8) has shown that the first rate limiting component for light emission in luminous bacteria extracts is reduced diphosphopyridine nucleotide. Later studies on this system have demonstrated that at least two other factors are required for light emission (9, 10, 11). It is clear, therefore, that the earlier operational definition of luciferin is concerned only with the first rate limiting factor for light emission in crude extracts. It is not necessarily a test for the molecule actually concerned in the final light emitting step. By comparing the biochemistry and physiology of the three luminous species discussed above, we believe that a clearer idea of the general problem of the luciferin-luciferase reaction will emerge. In the present review, however, the luminous bacteria will be discussed in greater detail since much of the information on the cell-free preparations has been obtained during the past year.

GENERAL THERMODYNAMIC AND PHYSICAL CONSIDERATIONS

Since the terminal reactions in bioluminescence are oxidative steps leading to electronically excited molecules, a consideration of the properties of and proposed mechanisms for chemiluminescent reactions is desirable. It is assumed that these latter reactions are fundamentally no different from those which require enzymes as catalysts.

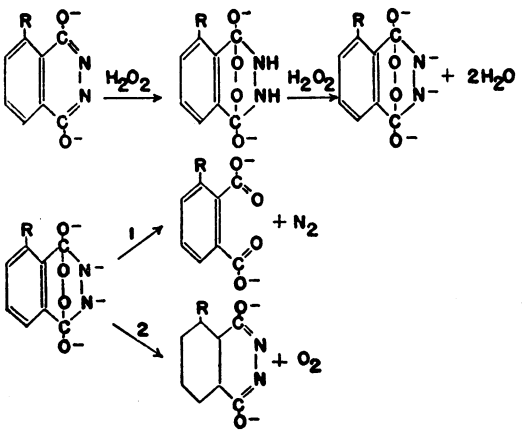

Figure 1. Proposed chemiluminescence reaction for 3-amino-phthalcyclohydrazide (13). Light emitted in reaction 2.

A molecule may be raised from a low, stable electronic state to an excited, unstable configuration by a variety of physical procedures (1). Thus, in addition to chemical reactions as a source of energy for the excited state of a molecule, thermal excitation may occur as, for example, in a wood fire or in the sun; the molecule may be excited through the absorption of a quantum of light (fluorescence); it may be brought into an unstable electronic configuration by the intervention of physical distortion particularly in crystals (triboluminescence); and it may be excited by means of an electrical discharge.

Chemiluminescence, however, is unique in that the excitation energy is derived from exergonic chemical reactions and in that it proceeds at an appreciable rate in solution at room temperature. Although it is impossible at present to describe the exact mechanism of chemiluminescence even for one single molecular type, the information which is available on the chemiluminescence of 3-amino-phthalhydrazide is reasonably complete. Chemiluminescent properties of this compound in the presence of peroxide, base and activator such as ferricyanide were discovered by Albrecht (12). The most definitive information bearing on the mechanism of luminescence of 3-amino-phthalhydrazide was furnished, however, by H. D. K. Drew, who in conjunction with his co-workers demonstrated an addition product between the chemiluminescent compound and peroxide (13). In figure 1 is illustrated the mechanism of chemiluminescence of 3-amino-phthalhydrazide as derived from the experiments of Drew. Essentially, these experiments showed that luminescence of the 3-amino-phthalhydrazide molecule could be blocked by substitution in certain positions but not in others. They also demonstrated that an intermediate addition product of the parent compound with peroxide could be isolated as a sodium or barium salt and that the treatment of this intermediate with oxidants (not necessarily peroxide) would give rise to a bright chemiluminescence. Drew postulated, therefore, that peroxide intervened twice in the chemiluminescence of this model compound. First, it formed an addition product, which subsequently was oxidized by another molecule of peroxide. He proposed that the

luminescent molecule acts as an intermediate catalyst for the degradation of peroxide and that the energy liberated arises from this reaction rather than from the destruction of the luminescent molecule itself. Drew suggested that 3-amino-phthalhydrazide was the acceptor of the energy from peroxide decomposition and acted as a mediator for energy liberation via light emission.

The importance of organic hydroperoxides has been emphasized by a number of workers in this field. Recently Linschitz and Abrahamson (14) have studied light emission from the reaction of zinc tetraphenylporphine (DH_2) and tetralin hydroperoxide ($>C(H)-O-O-H$). In this bimolecular reaction they could demonstrate the catalytic decomposition of the hydroperoxide by the porphyrin dye. They propose the following reaction mechanism:

$$>C(H)\cdot O\cdot O-H + DH_2 \rightarrow CO\cdot + DH\cdot + H_2O$$

$$>C(H)-O\cdot + DH\cdot \rightarrow DH_2^* + >C{=}O$$

$$DH_2^* \rightarrow DH_2 + \text{light}$$

The isolation of the ketone and the slow destruction of the dye during this reaction are evidence consistent with the proposed mechanism. In the case of bioluminescence the requirement of oxygen for light production may be used as an argument for a reaction analogous to the peroxididic chemiluminescence of synthetic organic compounds. The obvious difference between the two processes is that an enzyme is required for bioluminescence. The degree of analogy between *in vitro* chemiluminescence and bioluminescence remains an open question until the intermediates in both processes have been accurately defined. Although all arguments based on this information are tentative, nevertheless a consideration of experimental findings concerning chemiluminescence may furnish certain clues for the understanding of bioluminescence. The possible participation of peroxides in the latter process will be discussed in a later section.

The color of light emitted by two luminous species is shown in figure 2. The wavelength distribution of light emitted extends from the blue violet to the red region of the spectrum. Thus, approximately 40–60 kcal per M of quanta (Einstein) are required, and this, in the short wavelength regions, corresponds to slightly more energy than is available per two electrons at the average reduction level of carbohydrate.

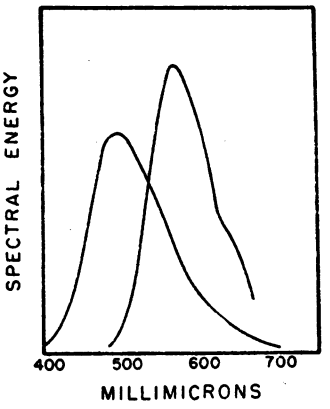

Figure 2. Spectal energy of light emitted from bacteria (left) and fireflies (right).

Most bacterial luminescences and the luminescence of extracts of *Achromobacter fischeri* center in the blue-green at approximately 500 mμ (2). The luminescence of *Cypridina* is a brilliant blue with a maximum at about 480 mμ, while the luminescence of firefly extracts and of the intact firefly shows a maximum at 565 mμ and a subsidiary peak at 620 mμ (15). Some minor differences are apparent in the gross emission spectra of different species of fireflies (16, 17). Whether this is a true difference in the emission spectrum of the chemiluminescent compounds involved or of a filter action on the part of the surrounding material is as yet not completely settled. Inasmuch as the luciferin obtained from the two species of fireflies can cross-react and both seem to possess similar physical and enzymological properties, it seems likely that the latter interpretation is correct (18). Macaire, in 1821 (19), showed that a heated preparation of firefly tails emits a much redder light just prior to the extinction of the reaction than is normally observed from the intact animal. Harvey (20) has suggested that the observed change is due to a difference in scattering at higher temperatures because of a partial denaturation of the surrounding structures. No such change in the color of the emitted light is observed when a mixture of the purified components is heated.

While earlier analyses of the emission spectra of luminous bacteria had indicated two fundamental frequencies (21), more recent work by Spruit and van der Burg (22), in which particular care was taken to avoid scattering and self-absorption, has demonstrated no dichotomy of fundamental frequencies but rather a single emission maximum at around 500 mμ. The wide variation in the spectral energy distribution of light emitted by different luminous organisms is further support for the idea that different luciferins are involved in these reactions. A comparison of the various luciferins will be made in a later section.

GENERAL PHYSIOLOGY AND BIOCHEMISTRY OF LUMINOUS BACTERIA

There are a large number of different luminous species of bacteria which are distributed among various genera, including the *Pseudomonas* and *Vibrio*, as well as the subgenus *Photobacterium* (2). The two luminous species which have been used most extensively for physiological and biochemical studies are *Photobacterium phosphoreum* and *Achromobacter fischeri* (*Bacterium phosphorescens indigenus*). Both are marine forms. Doudoroff (23) was the first to make a careful study of the fermentative metabolism of several species of luminous bacteria. He found that all of the facultatively anaerobic forms investigated showed the same general "mixed acid" type of anaerobic sugar dissimilation. Formic, acetic, lactic and succinic acids, alcohol, CO_2, and acetylmethylcarbinol (acetoin) were among some of the products formed. *Photobacterium phosphoreum* was capable of producing hydrogen. Recently Friedman (24) has demonstrated in cell-free extracts enzymes of the Embden-Meyerhof system as well as those associated with the glucose monophosphate shunt pathway.

Among the factors which have been found to influence bacterial luminescence are salt concentration, amino acids, carbon sources, and molecular oxygen. The work of Farghaly (25) and others has demonstrated that there is a rather critical optimum of salt concentration for bacterial luminescence and growth. The effects on these two processes are not identical: the process of growth is less inhibited by low salt concentration than is luminescence. Analysis of the inhibition of luminescence development in hypotonic media has shown that the effect is mainly osmotic in nature.

Although *Achromobacter fischeri* will grow on a minimal medium consisting of inorganic salts and glycerol as the carbon source, certain amino acids, notably methionine, histidine and lysine, increase the luminescence and decrease the lag in the growth. The nitrogen sources used most favorably by *A. fischeri* for growth and luminescence are the ammonium salts. The bacteria cannot use nitrate although they rapidly reduce nitrate to nitrite. Serine, glutamic acid, and guanine will support growth, but luminescence fails to develop. Both glutamine and asparagine will support growth and luminescence but are far inferior to the ammonium salts. Under conditions for optimum growth on ammonium salts Farghaly demonstrated that methionine plus histidine or methionine plus lysine would increase the luminescence 65 per cent without affecting growth. In the presence of all three amino acids the luminescence was lower than in the foregoing combinations. Methionine was the only amino acid which reduced the lag in the development of luminescence. Doudoroff (26) had also demonstrated that certain strains of luminous bacteria require methionine for growth and light production.

In the basal medium described by Doudoroff (23) and McElroy and Farghaly (27), growth and light production were normal at 25 C or less, but both failed to occur above 27 C unless hydrolyzed casein was added. Anderson (28) identified the amino acids in the hydrolyzed casein which were required for growth at the higher temperature as methionine, glutamic, aspartic and arginine. The elimination of any one of these amino acids almost completely prevented growth and light production. Serine could replace aspartic and arginine, provided lower concentrations of methionine and glutamic were used. The critical requirement of amino acids for the development of luminescence is illustrated by the mutational studies on luminous bacteria by McElroy and Farghaly (27). In a mutant requiring aspartic acid for growth, they found that luminescence failed to appear at a concentration of aspartic acid which gave nearly maximum growth. Higher concentrations of aspartic acid allowed the rapid development of the luminescent system. With an aspartic acid concentration which gave maximum growth, it was found that a mixture of methionine, arginine, leucine and isoleucine would completely restore luminescence. The elimination of any one of the four amino

acids from the mixture prevents the production of any significant amount of light, irrespective of the concentrations of the remaining 3 amino acids. The complexity of the relationship of amino acid requirement for growth and luminescence is illustrated by multiple mutants. McElroy and Friedman (29) illustrated that by crossing two mutants which required single amino acids, growth could be restored to normal by adding the two growth factors. The luminescence, however, was only 10–15 per cent of normal. Friedman (24) has demonstrated that a multiple mutant requiring arginine, proline, histidine, lysine, tyrosine and methionine for growth did not luminesce when grown on the minimal medium plus these amino acids. Luminescence developed normally, however, if the medium which supported growth was supplemented with aspartic acid (figure 3).

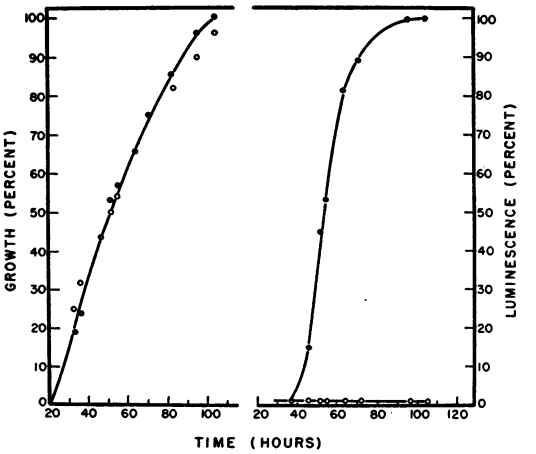

Figure 3. Effect of aspartic acid on light production in a multiple mutant of *Achromobacter fischeri*. Open circles refer to light intensity. Graph on the right represents growth and light production in the absence of aspartic while the graph on the left represents the effect of supplementation with aspartic acid (24).

Farghaly has studied a number of carbon sources for their ability to support growth and light production of *A. fischeri* (25). Of the 20 compounds tested, only glycerol and glucose were utilized by this organism for growth and luminescence. Friedman has since found that the bacteria can grow on fructose, provided small amounts of either glutamic acid, histidine, methionine or cysteine are added to the medium (24). A certain amount of growth occurs in the presence of low fructose concentrations, whereas higher concentrations completely inhibit growth. This amino acid requirement for fructose utilization remains unexplained. It is interesting that Farghaly has found that these same four amino acids are effective in initiating growth in a low CO_2 atmosphere. Friedman found that extracts of cells grown on glucose rapidly phosphorylate fructose in the presence of ATP, and that resting cell suspensions rapidly oxidize this compound. He has suggested that fructose or compounds formed from fructose may inhibit growth by reacting with free amino nitrogen in cells.

Other species of luminous bacteria have been studied for their ability to grow on a variety of carbon sources (see Harvey, 2). Johnson (30), in an extensive study, has shown that resting cells of *Photobacterium phosphorescens* and *A. fischeri* rapidly oxidize a variety of carbon sources. Most of these substrates do not support growth.

Doudoroff (31) in earlier studies found that added riboflavin was essential for maximum luminescence during growth. Interestingly, the respiration was the same in the bright and dim strains as in dim strains plus riboflavin. From these observations Doudoroff concluded that a flavin was connected with one of the enzymes involved in light production by the bacteria.

As a result of work with inhibitors and other indirect studies, a number of workers have suggested that the light emitting process of bacteria is coupled to the main respiratory pathway of these organisms (32, 33, 34, 35, 36). In Harvey's earlier studies (37) it was clear that the terminal oxidases were far more sensitive to the action of cyanide than was the luminescent reaction itself. Because of the suggestion that cypridina luciferin might be a naphthoquinone derivative (38, 39), Spruit and Schuiling (40) studied the effect of a variety of these compounds on bacterial luminescence. They found that the percentage inhibition of respiration was always lower than that of luminescence. They explained this inhibition of luminescence as an oxidation of luciferin whose redox potential was deduced to be -0.050 volts. Since addition of KCN relieved the inhibition of the naphthoquinones, Spruit and Schuiling believed the naphthoquinones were being reoxidized by a cyanide sensitive catalyst. As will be discussed later, the partially purified luciferase preparation does indeed rapidly reduce quinones and in so doing inhibits the light emitting reaction. McElroy and Kipnis (36)

were unable, however, to confirm the cyanide effect reported by Spruit and Schuiling. From kinetic studies on the inhibition of light by 2-methyl-1,4-naphthoquinone they concluded that at least two pathways of electron transport support luminescence. As a result of Friedman's studies (24) it seems possible that these two pathways are the Embden-Meyerhof glycolytic system and hexose monophosphate shunt. The latter pathway is apparently more sensitive to the quinones as far as supporting luminescence is concerned. In the presence of glucose and 4×10^{-6} molar 2-methyl-1,4-naphthoquinone luminescence is inhibited only 20 per cent. When, however, the glucose disappears from the medium, the light intensity rapidly drops to over 70 per cent inhibition. This is also true for the action of dinitrophenol. Kinetic analysis of luminescence under these two conditions indicates quite clearly that only one of the possible rate limiting pathways is supporting light emission. All of these studies support the idea that the light emitting system in luminous bacteria represents a by-pass of the cyanide sensitive portion of the cytochrome system. In addition, however, they suggest that the luminescent system is closely associated with the cytochrome system and, in fact, competes with the latter for electrons derived from the breakdown of food material.

BIOCHEMISTRY OF LUMINESCENCE

Bacterial Luminescence

Extraction of the system. Harvey's monograph *Bioluminescence* (2) has reviewed early attempts to extract the luminescent system from luminous bacteria. These pursuits were almost uniformly unsuccessful, due perhaps to insufficient sensitivity on the part of light measuring equipment and the lack of many important biochemical intermediates. Nevertheless, Gerretsen (41) in 1920 succeeded in obtaining a weak "luciferin-luciferase" reaction from extracts of *Photobacterium Javanense*. Unfortunately, Gerretsen did not pursue these studies, and other workers were unable to confirm his findings (2, 42).

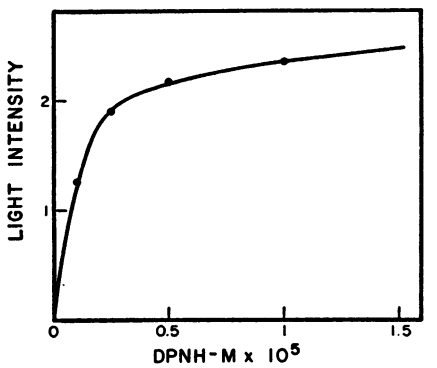

Figure 4. Relationship between light intensity of bacterial extracts and DPNH concentration (44).

In 1951 Shoup and Strehler (unpublished) noted that acetonized powders from *Achromobacter fischeri* would luminesce brightly for several minutes after being suspended in water. Boiled extracts of these same bacteria did not restore the luminescence after the cold water extracts had ceased to give light. Some dim luminescence did occur when hydrogen peroxide was added either to a cold water or boiled extract, but this is probably due to the peroxidation of riboflavin (43).

Components required. Following these observations, Strehler and Cormier (8, 10) undertook examination of the luminescence of acetonized *A. fischeri* powders. They observed that the duration of luminescence depends on the concentration of bacterial powders used and that a luciferin-luciferase reaction was obtainable if sufficiently concentrated extracts were employed. The factor which first became limiting for luminescence in the crude extract was shown to be diphosphopyridine nucleotide (DPN^+) or its reduced homologue (DPNH). The relationship between DPNH and light intensity is shown in figure 4. By the classical definition this compound was bacterial luciferin just as ATP is firefly luciferin in the classical sense for most species of fireflies. Initial attempts to demonstrate a requirement for other diffusible factors were inconclusive, in that prolonged dialysis and acetone fractionation gave preparations still capable of responding solely to added DPNH. However, certain cofactors, such as flavin mononucleotide, coenzyme A, and thiamin pyrophosphate, were effective in increasing the luminescence of crude extracts under various conditions. In addition, a potent stimulation of luminescence was obtainable from water extracts of defatted hog kidney cortex and other tissues. This material was called kidney cortex factor or KCF (10, 11).

In contrast to the failure of Strehler and Cormier to demonstrate an absolute requirement

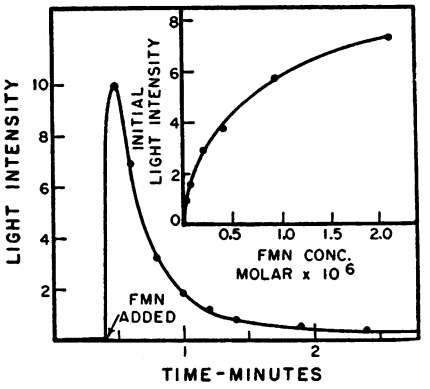

Figure 5. Relationship between light intensity of bacterial extract and riboflavin phosphate (FMN: flavin mononucleotide) concentration (9).

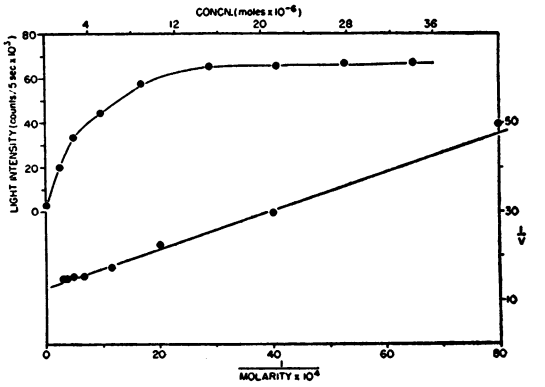

Figure 6. Relationship between light intensity of bacterial extracts and palmitic aldehyde concentration (45).

for a factor other than DPNH for the luminescence, McElroy *et al.* (9) were able to resolve distilled water autolysates of *A. fischeri* completely for FMN by a combination of acid precipitation and ultraviolet irradiation. Their preparations would not emit light in the absence of added FMN, thus indicating that the luminescent pathway consists of a DPN-flavin electron transport system (figure 5). McElroy *et al.* also observed a requirement for an additional factor which they termed bacterial luciferin. This factor was, in certain respects, analogous to the component in firefly luminescence which McElroy and Strehler have called firefly luciferin (5, 6). It now appears that this component is similar to KCF since it has no observable effect in the presence of excess KCF. Subsequently Cormier and Strehler (11, 45) identified the component in hog kidney cortex responsible for the marked increase in luminescence as the long chain aldehyde, palmital. This factor had been isolated in 1928 by Feulgen *et al.* (46) as a breakdown product of plasmolgen, the palmitic acetal of glycerophosphocholine. In the presence of KCF, DPN, malate, and FMN, it has been possible to obtain luminescence of cell and particle-free extracts of *A. fischeri* for 24 hours or more. The relationship between KCF and light intensity is shown in figure 6. Both the rate of luminescence and respiration at low oxygen tensions are increased by KCF (see figure 7).

Strehler *et al.* (47) have recently demonstrated that a bright luminescence will occur when reduced riboflavin or reduced FMN is added to

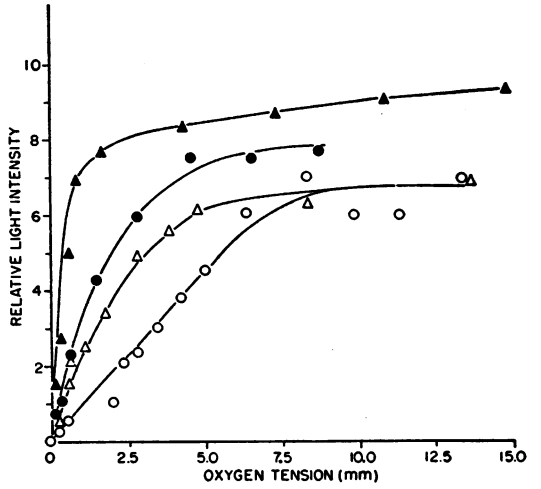

Figure 7. Effect of palmitic aldehyde on luminescence (triangles) and respiration (circles). Open circles and triangles are the results without aldehyde (45).

the bacterial enzyme and that the long chain aldehydes are also required for the optimal luminescence under these conditions (see figure 7).

Diaphorase properties. The partially purified system acts as a typical diaphorase (44). Both methylene blue and 2-methyl 1,4-naphthoquinone are rapidly reduced by the system. Even in the absence of the dyes, DPNH oxidation occurs at a reasonable rate only if FMN is present. Under normal oxygen tension the presence or absence of the aldehyde does not affect the DPNH oxidation in the partially purified preparations. It is possible, therefore, that much

of the DPNH oxidation is through a pathway which is not directly concerned with light emission. Ferricyanide, likewise, leads to a very rapid oxidation of DPNH. In fact, if sufficient ferricyanide is added to oxidize the DPNH, no light emission is observed from the reaction. On the other hand, with lower concentrations of ferricyanide the light emission is completely delayed until the ferricyanide is reduced, and then once this occurs, light appears.

Effect of various environmental factors and inhibitors. The general temperature dependencies of the bacterial luminescent reaction *in vitro* and *in vivo* are similar with an optimum at approximately 25 C (10, 48, 49, 51) (*A. fischeri*). Increasing the salt concentration of the reaction mixture produces first an increase, then at higher concentrations, a decrease in the rate of luminescence. The pH dependence of the reaction in crude extracts (10) exhibits a double optimum (pH 6 and 8.5), while in more purified preparations a single optimum is observed (44) at pH 7.0.

Gerretsen (41) observed an increase in luminescence after a short ultraviolet treatment, and this observation has been repeated by Harvey. Similarly, irradiation of the crude enzyme with ultraviolet light (365 mμ) produces an initial increase and then a decrease in luminescence (9, 10). This effect has been examined in some detail by McElroy and co-workers. By $(NH_4)_2SO_4$ fractionation it is possible to separate from the light emitting system a fraction which stimulates luminescence only after irradiating with ultraviolet light. It seems likely that the active material is similar to the long chain aldehyde. The partially purified luciferase is rapidly inactivated by the irradiation which probably accounts for the decrease in light intensity after prolonged ultraviolet treatment. The luminescent reaction is very sensitive to a variety of chemical agents. Riboflavin and flavin adenine dinucleotide inhibit probably by competing with FMN at the site of its reduction. The inhibition by *p*-Cl-Hg-benzoate, reversed by glutathione, and by copper and silver indicates the necessity of a sulfhydryl group for enzyme activity. The inhibition by versene and cyanide indicates the possible involvement of a metal; however, in the partially purified preparations consistent reactivation by metals after versene treatment has not been observed. The cyanide may be acting by combining with the aldehyde. It is interesting that cyanide should inhibit the isolated system, whereas it has very little effect even in higher concentrations on light emission in the intact bacterium.

TABLE 1

Time required for half-maximal luminescence in bacterial extracts when various essential components are added last

Component Added Last	Factors Present with Enzyme	"1/2 Rise Time"
		sec
Malate	DPN^+, FMN, KCF, O_2	108
DPN^+	Malate, FMN, KCF, O_2	108
DPNH	FMN, KCF, O_2	2.7
FMN	DPNH, KCF, O_2	2.7
$FMNH_2$	O_2	0.41
$FMNH_2$	KCF, O_2	0.075
RFH_2	O_2	0.27
RFH_2	KCF, O_2	0.14
KCF	FMN, DPNH, O_2	0.08
O_2	FMN, DPNH, KCF	0.05

The ½ rise time is the time required to reach ½ maximal light intensity.

A number of kinetic observations on the crude and FMN resolved luciferase preparations have been made (50). Particularly, the time course of luminescence has been followed when one of the necessary factors was added last, *e.g.*, DPN^+, malate, DPNH, FMN, O_2, KCF, $FMNH_2$, and reduced riboflavin. These results are summarized in table 1. Assuming that the shortest half rise time would be characteristic of later steps in a series of reactions, the following sequence has been postulated for bacterial extract luminescence.

$$\text{Malate} + DPN^+ \rightarrow DPNH$$
$$DPNH + FMN \rightarrow DPN^+ + FMNH_2$$
$$FMNH_2 + O_2 \rightarrow \text{light}$$

The effects of high hydrostatic pressures on the luminescence of cell-free extracts have been examined by Strehler and Johnson (51). Their results may be summarized as follows: When pressure is applied, there is an initial rapid increase in luminescence followed by an exponential decrease to some lower level. The magnitude of this effect depends upon the pressure, temperature and presence of KCF. Typical results are shown in figure 8. These results are explicable in terms of and consistent with the kinetic evidence obtained earlier. The rise, when pressure is applied, is regarded as the

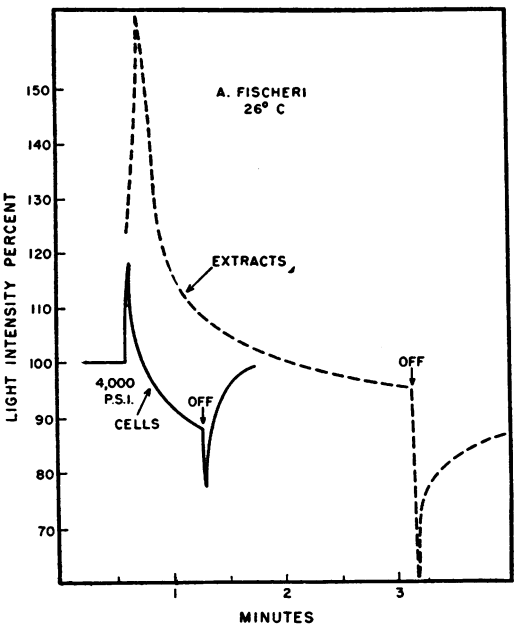

Figure 8. Effect of hydrostatic pressure on the light intensity of bacterial cells and extracts. Pressure was applied at 30 seconds and released at 75 and 195 seconds (51).

result of an increased efficiency of the luminescent oxidation of flavin at high hydrostatic pressures. The later exponential decline can be viewed as a depression in the pool size of $FMNH_2$ due to an inhibition of the DPNH-FMN reaction. The effects are generally similar to those observed in the intact bacterium. The tentative identification of the slow reaction as a readjustment of pool size was made by determining the ½ rise time when FMN, DPNH, enzyme and KCF were mixed. This value was compared with the time constant exhibited by the system in moving to a new steady state after the pressure had been altered. At 5 C the times were approximately 15 sec. The luminescent oxidation of substrate amounts of reduced flavin was accelerated by high pressures.

Firefly Luminescence

The production of light by extracts from fireflies has been shown to depend upon the presence of adenosine triphosphate, a highly fluorescent compound called luciferin, luciferase, oxygen and magnesium ions (4, 5). The luciferase has been purified from crude aqueous extracts by absorption on calcium phosphate gels followed

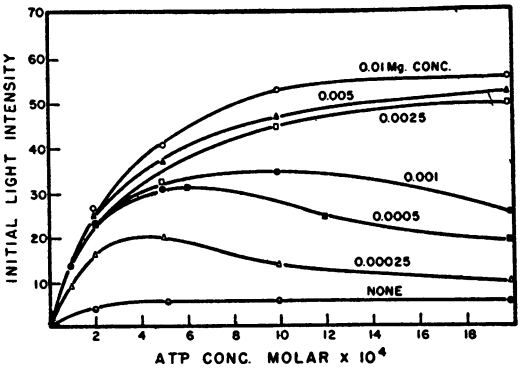

Figure 9. Relationship between light intensity of firefly extracts and adenosine triphosphate and Mg^{++} concentration (55).

by ammonium sulfate fractionation after elution (7, 52, 53). These preparations emit no light in the absence of ATP. The luciferin has been purified by extraction from the aqueous solution with acidified ethyl acetate and finally by column chromatography using "dowex 50", fullers earth-celite mixtures (52, 54) and celite, H_2O, chloroform-butanol columns. The properties of the luciferin will be discussed in a later section. In other species of fireflies such as *Photinus melanotis* or *Diphotus montanus* the crude aqueous extracts do not respond to ATP (18). To obtain light from these preparations it is necessary to add the purified luciferin obtainable from *Photinus pyralis*, as well as ATP.

The response of the purified system, obtained from *Photinus pyralis* to varying concentrations of ATP and Mg^{++} is shown in figure 9. The variation of the light intensity with these two components suggests that a complex between ATP and Mg^{++} is the effective substrate (55). This is particularly noticeable with low Mg^{++} concentrations where it is possible to demonstrate an inhibition with high ATP concentration. For the intermediate range of concentrations the maximal initial light response is obtained when the molar ratio of ATP to Mg^{++} is one. In the purified enzyme preparation light emission cannot be elicited by a variety of other phosphorylated compounds which have been tested. The initiation of light emission in the crude extracts by ADP is due to the presence of an effective myokinase (7, 56). Inosine triphosphate, uridine triphosphate, acetyl phosphate, creatine phosphate, inorganic pyrophosphate, and a variety of other phosphorylated and nonphosphorylated cofactors

fail to initiate light in the purified preparations. The reaction is apparently specific for adenosine triphosphate. It is cyanide and azide insensitive but can be inhibited by *p*-Cl-Hg-benzoate. The latter inhibition is reversed by glutathione. Light emission is extremely sensitive to a variety of amines, particularly hydroxylamine, while various benzimidazole and benzothiazole derivatives inhibit by competing with the luciferin (McElroy, unpublished).

When the purified firefly components are mixed, there is an initial high light intensity which rapidly declines to an intermediate steady state level of luminescence of several hours duration. Once the light intensity has reached the low baseline level, it is possible to show by direct chemical analysis that most of the ATP is still present. It is also available for the hexokinase reaction (7). If additional luciferase is added to the reaction mixture, however, a second flash similar to the original is obtained. The results indicate that all of the low molecular weight components necessary for light production are present and available for maximum light production.

The decline of luminescence after its initiation with ATP to the low steady state level is believed to be due to the reversible formation of an inactive complex from an active intermediate. The latter intermediate is presumably composed of four components as indicated in figure 10. Probably through a series of reactions the active intermediate is finally converted, in the presence of oxygen, to an excited state which subsequently emits light. Recently it has been shown that under anaerobic conditions formation of the inactive complex still occurs (57). When oxygen is readmitted to a previously anaerobic system, there is a flash of light which decreases within a second to the low baseline level of luminescence. This flash can be interpreted in a number of ways. It may be that under aerobic conditions the consumption of the active intermediate is so rapid as to prevent a true equilibrium between the active and inactive complexes. Under anaerobic conditions true equilibrium can be established, and the momentary flash where oxygen is readmitted is due to the rapid utilization of an accumulated active intermediate. The formation and accumulation under anaerobic conditions of a further product from the active intermediate indicated in figure 10 are possible. Consistent with this latter interpretation are the following earlier observations: Certain dried preparations of firefly lanterns will luminesce when moistened but fail to respond to ATP addition; moreover, powders of desiccated firefly lanterns boiled in butyl alcohol have been observed to exhibit the same phenomena. Such an interpretation does not necessitate the postulation of a slow equilibrium reaction between the inactive and active intermediates, as compared to the luminescent reaction which utilized this active intermediate.

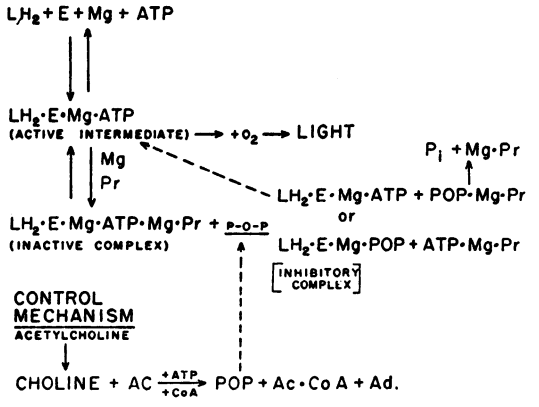

Figure 10. Proposed scheme for firefly luminescence.

POP: pyrophosphate
Pr: protein
Pi: inorganic phosphate

Although the formation of the inactive complex does not require oxygen, it is dependent upon a second protein. The removal of inorganic pyrophosphatase from the luciferase increases the steady-state level of luminescence, while addition of purified firefly or yeast pyrophosphatase depresses it. The results of such an experiment are shown in figure 11. The fact that inorganic pyrophosphatase is an essential protein in the formation of the inactive complex is important in explaining the effect of various pyrophosphates and polyphosphates on light emission. The addition of a variety of these compounds to the luminescent reaction temporarily restores the light to a high intensity. This action of inorganic pyrophosphate and triphosphate in stimulating light production, after its initiation by ATP, is attributed to the rapid breakdown of the inactive complex by these agents. In the case of pyrophosphate the evidence shows that it competes with ATP in formation of the active intermediate. Thus the addition of pyrophosphate before ATP strongly inhibits light emission. On the other

hand, the delayed addition of pyrophosphate leads to an initial stimulation followed often by a decline and then a rise to a secondary peak which rapidly decreases to the baseline level as the pyrophosphate is hydrolyzed by the action of pyrophosphatase. Such results are to be expected if pyrophosphate splits the inactive complex to form initially some active intermediate. The amount of active intermediate formed depends upon both the nature of the inactive complex(s) and the mode of the splitting by pyrophosphate. The simplest inactive complex presumably formed consists of luciferin-luciferase-Mg-ATP-Mg-pyrophosphatase. Pyrophosphate could split such a complex to give rise to either an active (luciferin-luciferase-Mg-ATP) or an inactive (luciferin-luciferase-Mg-pyrophosphate) intermediate containing luciferase (see figure 10). The initial light intensity obtained with the addition of pyrophosphate would then be a measure of the active intermediate formed. The secondary peak of luminescence following the addition of pyrophosphate would represent the slow release of luciferase from an inhibitory complex with pyrophosphate. The effect of various pyrophosphatase inhibitors, such as Mn^{++}, Ca^{++}, and F^{-}, on the luminescent response to pyrophosphate can be explained by such an hypothesis.

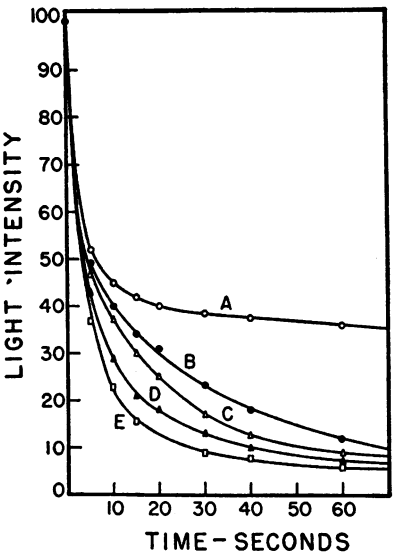

Figure 11. Effect of inorganic pyrophosphatase on the light intensity in firefly extracts. Curve A represents the reaction with purified luciferase. Curves B, C, D, and E represent the effect of adding increasing amounts of inorganic pyrophosphatase (55).

Cypridina Luminescence

The nature of the luminescent reaction in extracts from *Cypridina hilgendorfii* has been extensively reviewed by Harvey (2, 58). Therefore, only the more recent information will be presented for this system. Detailed studies by Harvey and associates indicate that only two components, in addition to oxygen, are required for luminescence, a luciferin and a luciferase (59, 60, 61). McElroy and Chase (62) have purified the luciferase over 150 times by $(NH_4)_2SO_4$ and acetone fractional precipitation and adsorption onto and elution from calcium phosphate gel. The luciferin has been partially purified through chemical procedures by Anderson, and by Mason using chromatographic procedures. The chemical and physical properties of the purified luciferin will be discussed in a later section. In general, the studies on cypridina luminescence indicate that only reduced luciferin, luciferase and oxygen are required for light emission. Moreover, it is not possible to obtain additional light in the crude extracts from dried *Cypridina* by adding other cofactors as is the case for fireflies and bacteria. Experiments on crude extracts from living *Cypridina* would be of comparative significance in this respect.

CHEMISTRY OF LUCIFERIN

It has already been pointed out and should be reemphasized that luciferin, being a component defined experimentally as a heat stable factor, is not necessarily the same molecule from species to species. Since luciferins obtained from various species do not generally substitute for each other in luciferase preparations from one species or group, it is certain that the luciferins can be of varying chemical natures.

Bacterial Luciferin

The recent demonstration of an absolute requirement for riboflavin phosphate in bacterial luminescence (9) as well as the luminescent oxidation of chemically reduced flavins (riboflavin and FMN) by bacterial extracts (47) strongly suggests that bacterial luciferin is reduced riboflavin phosphate. The former evidence establishes the luminescent system as a flavin coupled pathway while the latter dispenses with DPN as a necessary cofactor. Although the long chain aldehyde is necessary for luminescence, it

would seem a less likely candidate for the role of luciferin since it will not, by itself, support luminescence in the presence of the enzyme.

Certain facts are in opposition to considering reduced FMN as bacterial luciferin. Of particular importance is the fact that the fluorescence emission of riboflavin is centered at *ca.* 565 mμ, while the emission of the bacteria and extracts is maximal at 490 mμ. Second, one cannot rule out the possibility that some strongly bound compound other than flavin might be the ultimate acceptor and lumiphore. While these possibilities cannot be rigorously excluded, the following experimental observations are more consistent with a direct participation of reduced flavins in the luminescent reaction: (a) riboflavin and riboflavin phosphate will chemiluminesce in the presence of hydrogen peroxide (44); (b) if reduced flavins are oxidized by some other component, then this reaction must be accelerated by KCF (49) which from other experiments (50) seems to be involved in O_2 activation; (c) prolonged dialysis (10), repeated acid precipitation and ammonium sulfate fractionation (44) do not result in preparations of enzyme incapable of luminescing in the presence of DPNH, FMN and KCF; (d) if another factor is necessary, it not only must meet the requirements set forth above, but must not be destroyed on light emission since luminescence will continue for at least 24 hours in the presence of excess KCF, FMN and DPNH.

At the present time, in our opinion, the most reasonable view is that bacterial luciferin is reduced riboflavin phosphate or a derivative of the latter. It does not now appear that the inactivation spectra studies of the Delft group (63) and the tentative identification of bacterial luciferin by these workers as a naphthoquinone are in keeping with more recent direct biochemical findings.

Firefly Luciferin

Firefly luciferin has been demonstrated as an accessory factor for the luminescence of firefly extract supplemented with ATP, magnesium and oxygen. Strehler and McElroy (6, 52, 54) undertook to isolate and purify the active component. Liquid-liquid partition chromatography, paper chromatography, column chromatography, and other procedures were used to isolate a material of a reasonable degree of purity. Firefly luciferin is a faint yellowish compound having in alkaline solution a brilliant yellow-green fluorescence, resembling rather closely the fluorescent emission of riboflavin and firefly extract luminescence. However, in contrast to riboflavin, the yellow-green fluorescence disappears in acid solution. Microbiological assay also indicates the absence of riboflavin (McElroy, unpublished). In the strong acid solutions luciferin has a red-violet fluorescence, much weaker than its fluorescence in alkaline solution. At room temperature in acid solution it gives rise to another compound of similar but somewhat more yellow fluorescence, and with an absorption band shifted about 15 mμ to the red. This compound is enzymatically inactive. The pKa for the change in fluorescence and for the change in absorption spectrum is at about 8.4. Electrophoretic mobilities have been checked in agar tubes at various pH's using the fluorescence as an indicator of activity. It appears that the luciferin molecule is doubly charged in solutions more alkaline than pH 8.4, singly charged between pH 4 and pH 8.4, and that below pH 4 it is uncharged. These properties suggest an amine-imine isomerization and a carboxyl group. Further evidence for an active imine group was obtained by treatment by nitrous acid which destroyed both the fluorescence and enzymatic activity. The sensitivity of the reaction to imines may depend upon this property. The infrared absorption spectrum of luciferin, while differing greatly from the absorption spectrum of riboflavin in the 2 μ and 6 μ regions, shows a remarkable similarity to riboflavin in the 10–15 μ range. This evidence can be taken to indicate that luciferin has a basic nuclear structure closely resembling that of riboflavin in terms of mass. Polarographic analysis indicates that luciferin has an E_0' at about −0.6 volts at pH 7.0, while its degradation product has an E_0' approximately −0.4 volts. Riboflavin, on the other hand, has an E_0' at about −0.21. Using fluorescence changes as an index of the sensitivity of the compound to chemical reagents, the following was found: permanganate, peroxide, and hypochlorite treatment result in an inactive material.

Recently Strehler and Sites (64) have examined the mass spectrogram of pyrolyzed and unpyrolyzed luciferin. The largest mass obtainable, 293, may be taken as the mass of the parent compound. Large ion currents were obtained also at masses 278 and 234. An analysis of the complicated mass spectrogram has been undertaken

again using riboflavin as a model test compound. The mass 234 was taken as the parent nuclear mass, and considering the degradation product masses obtained, a structure for luciferin was proposed. This structure was subjected to test by the synthesis of some analogous compounds, whose gross physical properties approximated, in many instances, the properties of luciferin. The proposed structure is a dipyrimido pyrazine, doubly nitrogen and doubly oxygen or monosulfur substituted, with the carboxyl group pendent on the pyrimidine ring carbon. While it may be premature to assume that luciferin is actually a dipyrimido pyrazine related both to the pteridines and to riboflavin in general molecular architecture, further synthetic work should put this hypothesis to a rigorous test.

Cypridina Luciferin

Luciferin is the only known requirement for the luminescence of cypridina extracts, in addition to luciferase and oxygen. Nearly every investigator in the field has at one time or another presented a partial structure for cypridina luciferin based on various lines of chemical evidence. Among the proposed structures were a proteose (65), a phospholipin (66), a polyhydroxybenzene (67, 68), a reduced quinone, naphthoquinone or anthroquinone (38), and a flavin (69). The molecular weight of luciferin is probably between 250 and 500 mass units. Various purification procedures have been developed, some depending on extraction with organic solvents and fractional precipitation of impurities. Anderson (60) has obtained partially purified material by treatment of butanol extracts with benzoyl chloride to obtain the benzolyl derivative. The benzoylated material was then hydrolyzed and the cycle repeated. The activity per unit weight was increased over 2,000-fold by this double cycle. This luciferin has an absorption maximum at 435 mμ. Chase (70, 71) has demonstrated a shift in the absorption spectrum maximum from 435 to 465 mμ and then a gradual disappearance of the latter band as the luciferin undergoes autooxidation. Oxidized luciferin is colorless. Enzymatic oxidation of luciferin with accompanying luminescence is characterized by a similar change in absorption spectrum, but the effect here takes place at 100 times the rate of autooxidation.

Cypridina luciferin is capable of being oxidized both "reversibly and irreversibly" (2). If a preparation is exposed to air, it loses its ability to support luminescence when added to a luciferase preparation. But if within a short period of time the inactive material is treated with hydrosulfite or other appropriate reductants, its ability to support luminescence is restored. On the other hand, a long delay between the air oxidation and the re-reduction of the compound gives inactive material. Chakravorty and Ballentine (38) have presented evidence which may indicate that a ketohydroxy side chain is present in the active luciferin and that the irreversible oxidation involves the degradation of this substituent. They were able to regenerate active luciferin from irreversibly oxidized luciferin by a series of synthetic steps which were designed to introduce a ketohydroxy side chain onto the molecule. More recently Mason (72, 73) has shown through the use of paper chromatography under anaerobic conditions that several luciferins are present in cypridina extracts. He has called these *alpha* and *beta* luciferin. Acid hydrolysis of these luciferins followed by paper chromatography of the degradation products indicates the presence of a polypeptide. This polypeptide chain is believed to be attached to the chromophoric group whose chemical constitution is as yet undefined.

LUMINESCENCE IN OTHER FORMS: COMPARATIVE ASPECTS

Among the other luminous species on which some biochemical and physiological investigations have been made are the dinoflagellates in particular *Noctiluca*, the luminous ctenophore, *Mnemiopsis*, sea pansies, and several species of luminous earthworms (2). *Noctiluca*, which responds to physical stimulation by emitting light, should be an ideal organism for both biochemical and physiological studies. In this form, the luminous material is localized in granules. In the ctenophore, *Mnemiopsis*, the luminous material is localized in the region of the comb plates and likewise responds to mechanical stimulation as well as osmotic stimuli. This organism is remarkable in its apparent ability to luminesce under anaerobic conditions. Whether this indicates an entirely different chemical mechanism of luminescence from O_2 requiring organisms or not awaits the extraction and purification of the system. Buck (personal communication) has reported that sea pansies respond to ATP as do fireflies. This observation

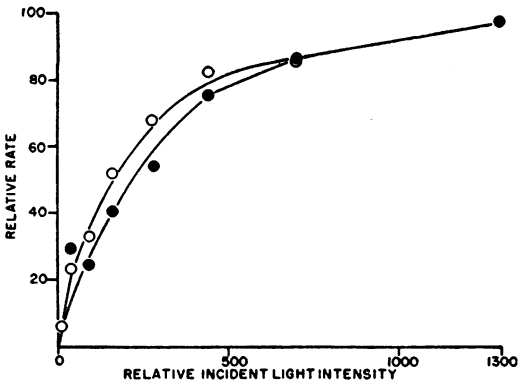

Figure 12. Dependence of luminescence and Hill reaction (open circles) rates of green plant chloroplasts on incident light (76).

suggests a biochemical resemblance between fireflies and a salt water form, which may be related to the fact that both are intracellular and under nerve control. The luminous earthworm, *Eisenia submontana*, on the other hand, reportedly utilizes riboflavin as a photophore and in this respect may be akin to bacteria (74).

The recent discovery by Strehler and Arnold (75, 76) of a chemiluminescence of low intensity given off by all green plants examined thus far adds this enormous group of living things to the plants and animals producing light through enzymatic oxidation. Despite the fact that this light is extremely dim, the total light emitted by green plants exceeds by many times the luminescence of all other living things. These studies have demonstrated a dependence for light emission on the photochemical and enzymatic apparatus of photosynthesis while the action spectrum in green plants and the luminescence emission spectrum are characteristic of chlorophyll absorption and fluorescence, respectively. In contrast to fluorescence (*i.e.*, immediate light emission), this bioluminescence saturates at higher light intensities with an enzymatic dependence. Strehler and Arnold have suggested that this luminescence is due to a recombination of early oxidizing and reducing intermediates in the photosynthetic process and have demonstrated a general dependence of luminescence on photosynthetic activity. Light energy absorbed by chlorophyll is thus converted into a more stable chemical form and then feeds back to regenerate excited chlorophyll in an enzyme catalyzed step. Photosynthesis and bioluminescence, ostensibly opposite processes, are thus directly shown to be the reverse of each other. In figure 12 are shown the dependence of the luminescence and Hill reaction rate of green plant chloroplasts on incident light intensity.

Considering the evolution of luminous species the singular ability to produce light would not, in our opinion, confer a great survival value on the organisms endowed with it since there are many more nonluminous than luminous forms. Secondarily, however, this ability may be adapted to uses which do confer a selective advantage on the luminous organism. In the primary category may be placed the luminous bacteria, while more highly evolved forms, such as fireflies, make use of luminescence in a secondary capacity.

In fireflies luminescence serves the social function of communication, males and females of the various species finding each other in their twilight haunts by means of a yellow flashing abdomen. One would hardly question the long range survival advantage of this unique ability. A variety of deep-sea forms apparently makes use of its luminescence in a less aesthetic and more immediate fashion, either by blinding of potential predators, attracting potential prey, or in the case of certain polynoid worms, distracting the predator from the more vital nonluminous portions of the organism. These worms, if attacked by a crab, reportedly luminesce brightly in their posterior portions, while the dark head end can crawl off and regenerate his lost tail. Luminous bacteria, on the other hand, probably do not glean any selective advantage under most conditions from their ability to luminesce. Rather their luminescence has been regarded as an accidental mutation in which the energy liberated by a terminal flavin autoxidase is channeled into the excited state of a molecule, probably flavin, which subsequently emits light. If in a natural environment this ability confers selective advantage, this advantage may reside more in side biochemical effects than in the physical phenomenon of light emission.

CONTROL AND MECHANISM OF LUMINESCENCE

In those species which do not glow continuously a number of mechanisms for producing and extinguishing the luminescence have been evolved. The most obvious device used is the interposition of an opaque body between the luminescent source and the outside. Thus a number of deep sea fish use either an eyelid-like or hinge-type flapper for shutting off the light

(2). In *Cypridina*, where the luminescence occurs outside of the animal, light production is initiated by the ejection of luciferin and luciferase, respectively, from two kinds of glands into the surrounding sea water (2). The control of the flash in fireflies has been the source of considerable speculation and controversy, some workers claiming that the light is initiated by admitting oxygen to the photogenic cells by neuromuscular control of the abdominal air ducts. McElroy and co-workers (55) have suggested, on the basis of the large amounts of pyrophosphatase in the luminous organ coupled with the light potentiating effect of pyrophosphate, that pyrophosphate release intracellularly and under neural control is the trigger mechanism (see figure 11).

The study of the time course of luminescence of bacterial, *Cypridina* and firefly extracts (9, 47, 50, 55, 77) when various necessary factors are added has furnished useful information concerning the mechanism of the luminescence. Typical time curve of the luminescence of these three species shows that there is an appreciable time-lag between the mixing of the components and the appearance of the maximum luminescence. It follows that some relatively slow process must precede the light emission step whose product is more directly concerned in the luminescent reaction. Chance, Harvey, Johnson, and Millikan (77) have discussed several possible interpretations of these observations. They have concluded that at least four reactions are necessary to explain the results including an association of luciferin with luciferase, an association of O_2 with luciferase, a reaction between O_2 and luciferin-luciferase, and a slow reaction leading to light emission.

Although the requirements for firefly extract luminescence are somewhat more complicated than the other two, a similar type of kinetic analysis can be applied to this system. Firefly luciferin is considered as analogous to *Cypridina* luciferin and reduced riboflavin phosphate in the bacteria. In the firefly, however, the luciferase requires specifically ATP for its activation of the luminous oxidation of luciferin, either as a component in an active complex between enzyme, ATP, luciferin, Mg^{++} and O_2 or as a reactant with luciferin to give a new active luciferin molecule. Another point of similarity among the various luciferins and one bearing on the mechanism is the fact that all of these compounds chemiluminesce with H_2O_2. With the above preliminary considerations and the work of Drew on 3-amino-phthalhydrazide chemiluminescences in mind, Strehler and Cormier (50) have suggested that an organic peroxide is involved in bacterial luminescence. In keeping with the suggestion is the observation that added peroxide stimulates firefly luminescence (McElroy and Coulombre, unpublished). Although the addition of peroxide or catalase was without effect on luminescence in bacterial extract, it is possible that in this system one is dealing with a strongly bound form of peroxide. One of the attractive features of this proposed mechanism is that it furnishes a means for supplying the 50 or 60 kilocalories which are necessary for luminescence. As pointed out previously, the catalytic decomposition of peroxide by nonenzymatic systems can supply the energy for chemiluminescence.

APPLICATIONS OF BIOLUMINESCENCE TO BIOLOGICAL PROBLEMS

The ability of a variety of chemical agents to elicit light from *in vivo* or *in vitro* luminescent systems has been the basis for a variety of analytical tests of high sensitivity, accuracy, and speed. Since the luminescent system is itself a respiratory pathway, the study of the effect of environmental variables on luminescence furnishes data on respiratory activity. Such effects as the flash of luminescence after anaerobicity in luminous bacteria indicate an accumulation of reduced intermediates in the absence of oxygen. Combined with inhibitor and pressure studies, the measurement of the light intensity and course of luminescence in luminous bacteria has furnished considerable information on the relation of luminescence to the main respiratory pathways and on the mechanism of enzyme action. Luminescence studies by Johnson, Eyring and collaborators have been concerned with the mechanism of action of various inhibitors and the types of bonds formed between inhibitors and enzymes.

One of the earliest examples of using luminous bacteria to solve biological problems was as oxygen detectors. Since light output is directly related to oxygen tension over a wide range of oxygen concentration, these organisms were applied to photosynthesis studies and furnished some of the earliest evidence that exogenous oxygen is not an obligatory requirement for the

initiation and maintenance of high photosynthetic rates.

Luminescence has also been applied more recently to photosynthesis problems of other types. The luminescence of green plants and its relationship to environmental factors have contributed to our understanding of early steps in photosynthesis while the use of firefly extracts for the measurement of adenosine triphosphate as applied to green plants has yielded information on both energetics and mechanism in phosphate dependent steps in photosynthesis.

Because of this unique ability of firefly extracts to emit light when ATP is added, this system is particularly useful in studies on energy transfer mechanisms. In combination with appropriate enzymes and substrates this system can and has been extended to many intermediates and enzymes affecting ATP levels. Among the substrates and enzymes of interest which can be assayed with firefly lantern extracts are ADP, phosphocreatine, AMP, glucose, coenzyme A, pyrophosphate, DPN, hexokinase, myokinase, creatine-adenylate transphosphorylase, and various apyrases and ATPases. In combination with ultra sensitive light detecting apparatus of moderate cost the sensitivity of the tests can easily be extended to the millimicrogram range. Among the problems to which this assay method has been applied are x-radiation effects on ATP metabolism, changes in ATP during grasshopper embryogenesis, oxidative phosphorylation, "ATP content" of purified nucleic acids, effects of narcotics on brain and bacterial ATP, and DPNase activity of brain tissue. The extracts from luminous bacteria also promise to be useful in the assay of DPN, its reduced homologue, and flavin mononucleotide.

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